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Title: Nucleotide Composition of the RNA of the Planarian, Dugesia dorotocephala, in the Normal and Regenerating State.

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In spite of the usage of the fresh water planarians for a number of interesting experiments on the mechanisms of morphogenesis (cf. Child, 1941), regeneration (cf. reviews by Brønsted, 1955, and Wolff, 1962) and learning (cf. reviews by Jacobson, 1963; McConnell, 1966), the basic anatomical, physiological, and biochemical information has been insufficient for elaboration of these mechanisms. Recently we have made some progress in this direction (Best, Rosenvold, Souders, and Wade, 1964; Morita and Best, 1965, 1966; Best and Elshtain, 1966; Best, Elshtain, and Wilson, 1967; Morita, 1965; Best, 1967). In view of the demonstrated role of ribonucleic acid (RNA) in the information transcription from DNA and translation in protein synthesis, it might be anticipated that the axial gradient in morphogenetic potential which has been demonstrated in planarians, as well as the cellular differentiation changes which must accompany regeneration, would find expression in differences of the RNA which must control these processes. In keeping with such concepts, Clement (1947) and Lindh (1953) reported alterations in the amount and base composition of RNA in regenerating planarians. Henderson and Eakin (1961) found irreversible alterations of their differentiated tissues to be produced by purine analogs. Corning and John (1961) found that ribonuclease treatment of planarians regenerating a new head abolished the effects of previous conditioning in the regenerate worm.

The studies to be described in this paper addressed themselves to the question of whether there is, in fact, any difference in the base composition of the RNA from head and tail regions and whether these undergo any alteration during regeneration of the worm.

METHODS

The planarians used in these experiments were Dugesia dorotocephala approximately 15 mm. long that had been maintained in colonies of approximately 500 in white enamel dishpans in the dark, or dim illumination, at a temperature of 70± 1° F. Prior to their use as experimental subjects they were fed twice weekly on raw beef liver. Following each feeding all food particulates were removed and the water completely changed. The water in which the planarians were maintained was tapped from the upper regions of a large aquarium tank with a substantial growth of green algae on its walls and a layer of crushed limestone through which the water of the tank circulated. This water, henceforth referred to as "M-water", yielded the analysis given in Table 1 and had been found to maintain colonies of these planarians in a state of good health and growth for periods of a year or more. The analysis given in Table 1 provided no information regarding trace elements and it is not known which of these are present or essential.

Table 1

The worms to be used as experimental subjects were fasted for 5 to 7 days prior to the beginning of the experiment. 100 worms were used for each experimental replication. 50 of these were sectioned by a transverse cut at the midpharyngeal level and place in the M-water. 50 uncut worms of the same size were also placed in the M-water at the same time.

At the end of 24 hours the regenerating pieces, as well as the uncut worms, were rinsed in fresh M-water and the uncut worms sectioned by a transverse midpharyngeal cut. All four groups of pieces (normal-anterior, normal-posterior, regenerating-anterior, and regenerating, posterior) were transferred to pieces of parafilm for determination of the wet weight of tissue.

For extraction of the RNA a modification of the procedure of Kirby (1962) was employed. A 0.5% solution of napthalene disulfonate (NDS) in distilled water was adjusted to pH 7 by addition of 0.01N NaOH. A phenol solution (P) was prepared by addition of 10 ml. of distilled water and 0.1 gram of 8-hydroxyquinoline to 90 grms of distilled liquid phenol. Each group of worm pieces were homogenized in a glass tissue grinder with 2 ml. of solution P and 4 ml. of NDS solution. The homogenates were transferred to 15 ml. Corex centrifuge tubes and centrifuged at 15,000g for 10 minutes. This procedure produces a separation of the phenol and aqueous phases into two distinct layers. The upper aqueous phase was pipetted off and reextracted with 2 ml. of solution P. This process was repeated until the interfacial region between the two phases was clear. Following the final removal of the aqueous layer, 2 volumes of cold 95% ethanol were added to this aqueous solution and the resultant solution allowed to stand overnight at 0° C. Following this overnight precipitation the mixture was centrifuged for 10 minutes at 15,000g and the alcohol - NDS supernatant solution decanted from the pellet of precipitated RNA. This RNA pellet was washed several times with large volumes of cold 70% ethanol, resuspended in 70% ethanol, and resedimented into a pellet by centrifugation. The RNA pellet was then dissolved in 0.2 ml. of o.3N KOH and allowed to hydrolyze overnight at 37° C. Slightly over 0.01 ml. of 28% perchloric acid was added to this hydrolysate to yield a pH of approximately 2 or 3. This mixture was then centrifuged at 15,000g for 10 minutes to sediment the precipitate of potassium perchlorate and any DNA which may have been present in the original extract.

The nucleotides in this supernatant solution were then resolved by a modification of the thin layer electrophoresis method of DeFilippes (1964). To do this an aliquot of the supernatant solution was measured out with a microliter syringe and spotted onto a thin layer strip (Brinkman MN polygram, Cel 300/UV)

4 cm. from one end. This thin layer strip utilizes a cellulose medium which is supported on a plastic backing and contains a fluorescent indicator. The strips were then sprayed with a citric acid - sodium citrate buffer (pH 3.3), put into an electrophoresis tray (Gelman model 51170) with more of the same buffer, and subjected to voltage gradients of 20 volts/cm. for 4 - 5 hours. The positions of the spots corresponding to the 4 nucleotides were located and marked on the dried strip by examination under an ultraviolet light source. These occur in the following order from the cathode: cytidylic acid, adenylic acid, guanylic acid, and uridylic acid.

The cellulose of each of these regions corresponding to the nucleotide spots was then quantitatively scraped off the plastic backing and placed in a centrifuge tube with 2 ml. of 0.2N HCl for 24 - 48 hours, to elute the nucleotides. A blank control thin layer strip was treated in the same way (including subjection to electrophoresis) as those containing the nucleotides and corresponding regions scraped off and eluted in the same manner. The amount of nucleotide was determined by measuring the U. V. absorbancy at 260 millimicrons in a U. V. spectrophotometer and using the specific molar absorbancy values for each nucleotide from the literature.

This complete experiment was replicated, as described, three times. An additional replication was conducted on regenerating worms with nonregenerating control groups as well as regenerating ones. Thus 350 planarians were used in these experiments described above.

For determination of the relative amounts of ribosomal and transfer RNA's, the RNA which had been precipitated and washed with cold 70% ethanol was redissolved in 0.05 M sodium phosphate buffer (pH 6.7). This was eluted through a G-100 Sephadex column equipped with a flow through cell and U. V. monitor. The column was previously equilibrated with the pH 6.7 buffer. The U. V. monitor was used to determine which tubes of the fraction collector contained the nucleic acid fractions. The amount of nucleic acid in each of

these fractions was determined by reading the optical densities of the relevant solutions in a U. V. spectrophotometer (Zeiss).

RESULTS

Table II shows the mole percent of each of the four nucleotides comprising the RNA of the head and tail regions of normal and regenerating planarians of the species, D. dorotocephala. The standard errors of these estimates are also shown. It is evident from a comparison of the columns of Table II that there is no significant difference in the base composition of the RNA's extracted from the head and tail regions of normal, i. e. unregenerating, planarians. It is also clear from such inspection that there is no significant difference between the base composition of the RNA's from the normal and regenerating planarians.

Figure 1 shows the optical densities at 260 millimicrons of the various fractions for elution of a sample of the planarians RNA through a Sephadex G-100 column. The large initial peak is a higher molecular weight RNA that is probably mostly ribosomal RNA while the smaller second peak is a lower molecular weight RNA that is probably mostly transfer RNA. The amount of ribosomal RNA (area under the large peak) comprises 80% of the total. The transfer RNA (area under the smaller second peak) is only 16.5% of the total RNA.

Table 2

Figure 1

DISCUSSION

The overall base composition of the RNA does not change between anterior and posterior regions nor between regenerating and nonregenerating planarians of the species, Dugesia dorotocephala, employed in these experiments.

This result is contrary to that reported by Lindh (1957) as well as to the expectations outlined in the introduction of the present paper. An explanation for this negative result is evident from the Sephadex column resolution of the ribosomal and transfer RNA's. A change in the base composition would be anticipated in the messenger RNA but not in the ribosomal RNA. If a drastic change in amino acid composition of the proteins being synthesized were to occur one might obtain a different distribution of the transfer RNA and obtain a different resultant base composition of the transfer RNA fraction. However, these would probably not produce much change in the overall base ratios. In other organisms, e. g. bacteria, approximately 80 to 85% of the total RNA is ribosomal, about 10 to 15% is transfer, and only about 3 to 5% is messenger. The relative sizes of the two major RNA peaks obtained in our Sephadex column resolution indicated that the ratio of transfer to ribosomal RNA in this species of planarian is about the same as that found in the bacterial systems.

It would, therefore, not be unreasonable to expect that messenger RNA in these planarians would comprise about the same proportion of the total RNA as it does in bacteria. If this is the case, then even large changes in the base composition of the messenger RNA would be obscured by the relatively large amount of ribosomal RNA in which the base ratios remain constant. In view of these considerations the differences in base composition reported by Lindh (1957) for the total RNA are somewhat surprising and need to be confirmed.

SUMMARY

RNA was extracted from anterior and posterior pieces of both normal and regenerating planarians (D. dorotocephala) by the phenol extraction procedure. This RNA was precipitated and washed with ethanol, hydrolyzed with KOH, and the hydrolysate fractionated into constituent nucleotides by thin layer electrophoresis. The resolved spots corresponding to each of the 4 nucleotides were eluted into solution. Amounts of each nucleotide were assayed from their U. V.

absorption. An unhydrolyzed sample of RNA which had been extracted from Dugesia dorotocephala by this phenol procedure, and purified in the same way, was resolved into its transfer and ribosomal fractions by elution through a Sephadex G-100 column.

No change was observed in the overall nucleotide composition of the RNA between anterior and posterior pieces or between regenerating and normal planarians. This negative result, which is contrary to the earlier results reported by Lindh (1957) on other species of planarians, can be accounted for by the preponderance of ribosomal RNA which maintains constancy of base composition.

TABLE 1

Results of Analysis of M Water

Characteristic	Amount in ppm
Total Alkalinity	16
Total solids	64
Organic matter	6
Calcium	32
Magnesium	1
Iron	0.01
pH	7.1

TABLE 2

Mole Percent Nucleotide Composition of the RNA in the Anterior and Posterior Regions of Regenerating Dugesia dorotocephala

	Normal Anterior	Normal Posterior	Regenerating Anterior	Regenerating Posterior
Cytidylic A.	17.4±.8	18.0±.8	17.7±.7	18.4±.7
Adenylic A.	27.7±.7	26.0±.7	27.3±.6	26.7±.6
Guanylic A.	32.0±1.5	31.3±1.5	30.2±1.4	30.8±1.4
Uridylic A.	23.5±.8	24.4±.8	24.7±.7	24.2±.7

Figure Legends

Figure 1: Resolution of the ribosomal and transfer components of planarian RNA by elution through a Sephadex G-100 column.

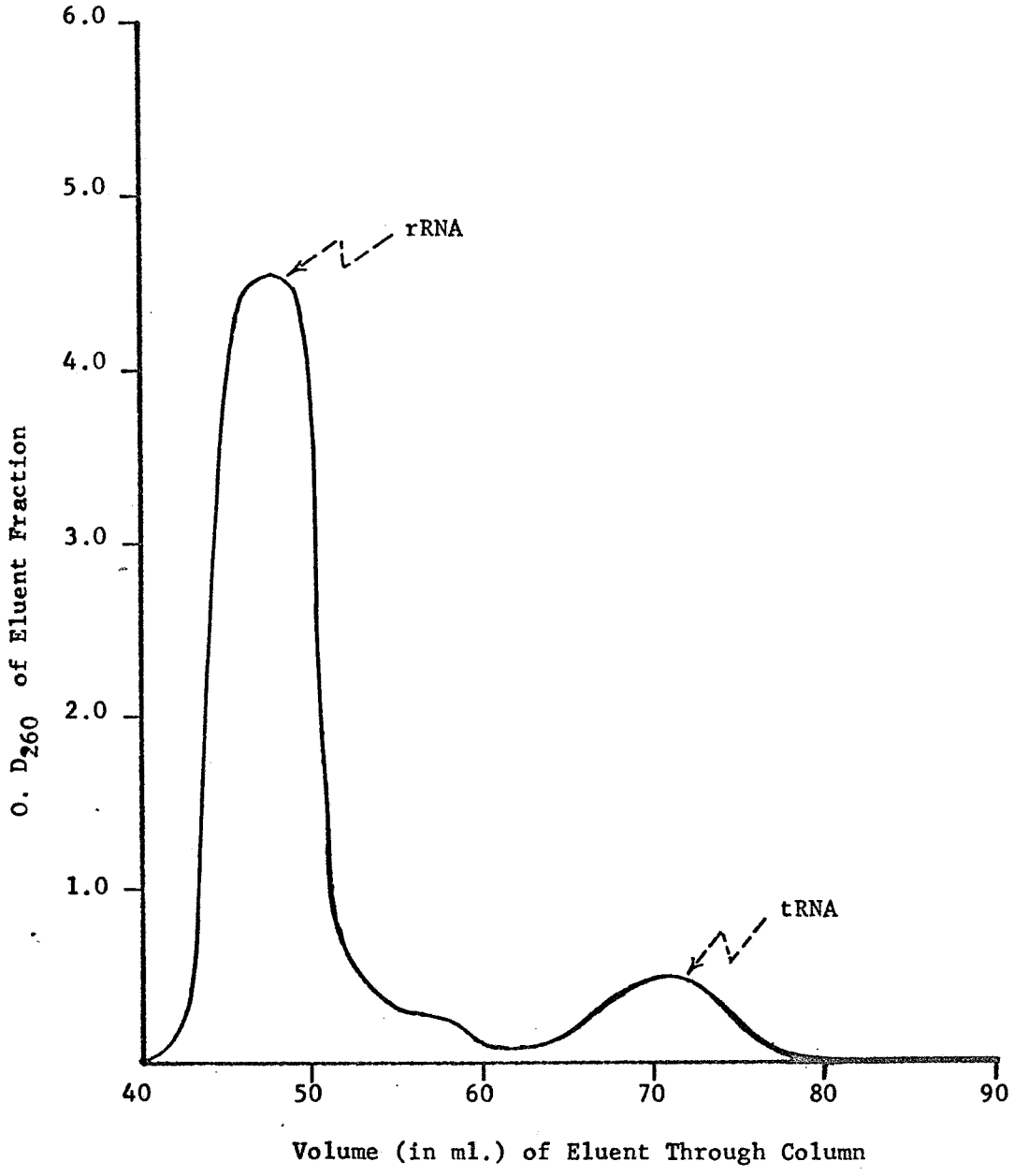
6.0
5.0
4.0
3.0
2.0
1.0
O. D_{260} of Eluent Fraction
rRNA
tRNA
40
50
60
70
80
90
Volume (in ml.) of Eluent Through Column

Figure 1

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